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<p>(54) Title: PROLIFERATIVELY ACTIVE PRODUCT FOR TREATING PROLIFERATING CELLS</p> <p>(57) Abstract</p> <p>A product comprising a proliferatively active moiety, typically a cytokine or growth factor which has a high affinity receptor, linked to a biologically active agent which agent preferentially or selectively affects proliferating cells. An active promoter of proliferation, for example an active IL-2, is beneficially used to deliver pharmacologically desirable species to cells whose proliferation is not desired. For example some medicaments of the invention control or inhibit proliferation using a molecule which contains an active promoter of proliferation. The biologically active agent is usually selected from the group consisting of antiproliferative drugs, compounds which interfere with nucleotide synthesis, radioisotopes, gene sequences and antisense nucleotide sequences.</p>			

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PROLIFERATIVELY ACTIVE PRODUCT FOR TREATING PROLIFERATING CELLS

5 The present invention relates to the use of proliferatively active compounds, especially cytokines or growth factors, as active vectors for pharmacologically active compounds, for example conventional drugs or genes.

10 Proliferation, differentiation and functional activity of most cells, particularly haematopoietic and immunological cells, are regulated by proteins called cytokines and growth factors. Separation of the two groups is difficult, due to several overlapping mechanisms and effects on target cells. For example, the cytokine Interleukin-2 is also known as T-cell Growth Factor. Cytokines and growth factors are both peptide hormones.

15 More specifically, cytokines regulate the functional status of their target cells (i.e. they can stimulate or suppress both quantitatively and qualitatively), whilst growth factors are more focused on promotion, regulation and maintenance of proliferation and differentiation, and the survival of their target cell lineages.

20 Both cytokines and growth factors recognise specific membrane receptors on their target cells, which are unique for that particular cytokine or growth factor. Each receptor, in turn, can express a dynamic avidity towards its specific cytokine or growth factor, based on physiological and/or pathological conditions. These receptors can be categorised as low, medium, or high affinity. Most important of all, high affinity receptors only recognise, capture, and internalise their related cytokine or growth factor. These receptors and their ligands are discussed in more detail below with reference to cytokines.

25 Cytokines are a group of molecules, other than antibodies, which are produced by lymphocytes and involved in signalling between cells of the immune system, for the purpose of stimulating or suppressing cell function. Cytokines often mediate their action by specific receptors expressed on target cells. Cytokines are glycosylated or non-glycosylated polypeptides and can be secreted by both T-cells and B-cells, though T-cells are assumed to be the major source in cell-mediated responses. Complications in the
30 study of cytokines have arisen from the fact that *in vivo* no cytokine ever operates in isolation. This is illustrated by the observation that many cytokine actions are synergistic. Important cytokines include interleukins (ILs), tumour necrosis factors (TNFs) and interferons (IFNs). In addition, various colony stimulating factors (CSFs) are secreted by myeloerythroid cells.

35 Receptors for numerous cytokines have now been cloned, and their structures (amino acid sequences) analysed. As a result, it is possible to group many of these into super families, based on common homology regions on their primary structures. For the purpose of this invention, the main super families recognised are cytokine receptor super family (CKR-SF), sometimes called haemopoietic receptor super

family, and the interferon receptor super family (IFNR-SF), also termed cytokine receptor super family Type II (Ref 1). The term "super family" should be used only to describe proteins with amino acid sequence homology of 50% or less. Proteins, with amino acid sequence homology of greater than 50%, are designated by the term "family".

5

Many cytokine and growth factor receptors have combinations of different domains or repeats. A domain is a sequence or segment of a protein which forms a discrete structural unit, able to capture and/or convert specific signals. For the purpose of this invention, the domains of interest are the extra-cellular regions (those located at the surface of a given target cell-lineage). Studies focussing on receptor binding have 10 revealed the existence of more than one binding affinity for several members of the CKR-SF (or haematopoietic receptor super family). Typically, these sites have low (e.g. 1-10nM) or high (e.g. at least 1pM and more usually 10-100pM) affinity to a given ligand (cytokine or growth factor). For most of these receptor complexes, additional sub-units have been identified which are required for high affinity receptor expression. These sub-units (also referred to as affinity convertors or convertor chains) are often expressed 15 on the cell surface after a given activatory or inhibitory stimulus is applied through a receptor ligand. This results in an amplification of effects, but only in those cells bearing the high affinity receptor and not in resting cells (which usually bear the low-active receptor complexes), and is the physiological basis of any paracrine stimulation/inhibition, in the absence of any involvement of district/regional/systemic networks.

20 Thus, to mediate immune responses, T-cells must change from a resting to an activated state. T-cells stimulated by foreign antigens enter a program of cellular activation leading to *de novo* synthesis of IL-2. Resting T-cells do not express high affinity receptors but these are rapidly expressed after activation. Interaction of IL-2 with its induced cellular receptors triggers cellular proliferation culminating in the emergence of effector T-cells that are required for the full expression of immune responses. Taking the 25 example of IL-2/IL-2r complex, in many of the diseases described herein, this physiological tuning is disrupted (primarily by neoplastic transformation, secondary to viruses), or is automaintained (autoimmune reactions/diseases, transplant rejection), leading to systemic multi-organ failure.

30 Further information about cytokines and their receptors may be found in Callard R E, Gearing A J H, The Cytokine-Factsbook, Academic Press - Harcourt Brace & Company, Publishers, 1994, 18-25.

High affinity receptors therefore include those with an affinity constant of $10^{-10}M$ or less, and, more particularly, those with an affinity constant of $10^{-11}M$ or less. Representative high affinity receptors include those with affinity constants of between 10^{-11} and $10^{-12}M$. For example, three forms of receptor for 35 interleukin-2 (IL-2) can be distinguished on the basis of their affinity for IL-2 with IL-2 binding affinities of $10^{-11}M$ (high affinity), $10^{-9}M$ (intermediate affinity) and $10^{-8}M$ (low affinity) (Refs 1-4). IL-2 receptors are well described in the prior arts (Refs 5 & 6).

TNF- α has been described as having two isoform receptors with high affinity on the target cells for TNF. These target cells are macrophages and osteoclasts (Ref 7). M-CSF (macrophage colony stimulating factor) has a high affinity receptor on macrophages and osteoclasts. The high affinity receptor is a 150 Kda glycoprotein (Ref 8).

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High affinity receptors have been described also for IFNs (interferons). IFN- γ has a 90 KDa glycoprotein as a high affinity receptor. A different receptor present on activated lymphocytes, macrophages, endothelial cells and fibroblasts has been recognised as the high affinity receptor of IFN- α and IFN- β (Ref 9).

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In the case of FGF (fibroblastic growth factor), there is a high affinity receptor which is a 140 KDa glycoprotein on mesodermic and neuroectodermic lineage cells, such as activated fibroblasts, macrophages, endothelial cells, chondrocytes, astrocytes, glioma cells, hepatocytes, epithelial cells, neurones, ovarian cells, pituitary cells, and keratinocytes. The pharmacological properties of FGF are primarily related to 15 angiogenesis, ovarian steroidogenesis, osteoblast activation, and nerve growth (during the foetal phase) (Ref 10).

IGF (insulin-like growth factor) has a high affinity receptor on eterotetrameric complex present in different tissues and in mammary adenocarcinoma (Ref 11).

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Transforming Growth Factor β (TGF β) is similar to IGF. TGF β is a non-glycosylated homodimeric protein secreted by fibroblasts, epithelial cells, platelets, astrocytes, monocytes, bone cells, and glioblastoma cells. The physiological target cells are primarily fibroblasts, osteoblasts, neutrophils, hematopoietic progenitors, T/B lymphocytes, and a range of tumor cells. The cytokine interacts with a high 25 affinity receptor, expressed by the target cells, in response to paracrine microenvironmental stimulation, located on the cell surface of the above cells. These are type 1 or type 2 receptors (55 and 80 Kda), and are able to bind to TGF β 1, 2, and 3.

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GM-CSF (granulocyte/macrophage colony stimulating factor) and SCF (stem cell factor) possess a dimeric high affinity receptor in multipotent cells in the bone marrow (Ref 12). G-CSF (granulocyte colony stimulating factor) also has a high affinity receptor present, but only in multipotent cells in the bone marrow.

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EPO (erythropoietin) has a multimeric high affinity receptor present on erythroid precursors in the bone marrow.

IL-6 (interleukin-6) has an α -/ β - high affinity receptor. The alpha chain binds IL-6 with low affinity and exists in a soluble form. The beta chain is a 130 KDa protein which simultaneously binds IL-6/IL-6r.

becoming a trimeric complex which initiates target cell stimulation. IL-6 high affinity receptor induction, following specific stimuli, is primarily positioned on activated cells such as T/B lymphocytes, fibroblasts, myeloid precursors, neurones, keratinocytes, and hepatocytes. In addition, multiple myeloma cells produce IL-6, and express IL-6 receptors working as an autocrine cancer growth factor, inducing at the same time 5 osteoclastogenesis (bone lytic lesions). IL-6 from stromal cells can also be involved in bone metastatic lesions through different tumour histotypes.

Several studies (in vitro, animal models, and humans) have been conducted using radiolabelled interleukin-2. The recombinant form of the protein was labelled with iodine-123 by the chloramine-T method (for 10 preclinical trials), or the lactoperoxidase-glucoseoxidase method (for clinical trials). The iodine-labelled cytokine was purified by an RT-HPLC technique in order to separate radiolabelled cytokine from non-radiolabelled cytokine.

Radio-labelled studies were carried out to evaluate the in vivo distribution of very-low dose radiolabelled 15 IL-2, when administered intravenously (iv) in pathological conditions in which naturally activated lymphocytes are the major factor responsible for the pathogenesis of a disease (Refs 13, 14). The models tested were autoimmune diseases, such as autoimmune diabetes mellitus (in diabetes-prone rats), patients with newly diagnosed autoimmune Type I diabetes, patients with newly diagnosed autoimmune thyroiditis, and patients with newly diagnosed chronic inflammatory diseases of the bowel (Coeliac disease and 20 Crohn's disease) (Refs 14, 15, 16).

The results from these studies have shown that:

1. The in vivo administration of very-low doses of labelled IL-2 is feasible without side-effects, both in animals and in humans.
2. After the distribution phase, labelled IL-2 is detected and retained only in those sites (organs) which are the target of the particular autoimmune pathological condition, and NOT in normal organs (i.e. in organs unaffected by the immune system attack).
3. Labelled IL-2 retains its capacity for binding to the IL-2 receptor on activated lymphocytes in vitro.

Other in vitro studies on lymphocytes have clearly shown that, following binding between IL-2 and its high 35 affinity receptor, the whole complex (IL-2/IL-2 receptor) is rapidly internalised into the cytoplasm of the lymphocyte. This process is fast (5 to 10 minutes), and effective only if the isoform receptor has the highest affinity with IL-2. After this internalisation, the endosome in the cytoplasm becomes progressively acidic (the pH is reduced to 5 through the action of some proteins on the membrane of the endosome which

work as proton pumps), and the two molecules (IL-2 and its receptor) are split to achieve their effects separately. (Ref 1)

- 5 A fusion protein of an IL-2 sequence and a diphtheria toxin sequence has been used to selectively target cells bearing the high affinity IL-2 receptor. The approach taken was to construct a diphtheria toxin-related IL-2 fusion gene that encodes a recombinant toxin in which the diphtheria toxin receptor binding domain is replaced with amino acids 2-133 of IL-2. The chimeric IL-2-toxin was expressed in recombinant strains of *E. coli* K-12 and shown to selectively inhibit protein synthesis in only those T-cell lines which express the high affinity IL-2 receptor (Ref 22). Similarly, it has been reported that a chimeric protein ("IL-2-PE40")
10 containing sequences of *Pseudomonas* exotoxin (PE) and IL-2 was able to provide a lymphokine-mediated method of delivering the toxin to IL-2r-expressing cells (Ref 6).

- 15 The chimeric IL-2-diphtheria toxin does not contain a biologically active IL-2 sequence but only the receptor-binding sequence. The protein has entered Phase III trials for the treatment of cutaneous T cell lymphoma (*Scrip*, January 13, 1995 and *New Scientist*, November 1, 1997). Blast leukemic cells usually bear only the low/intermediate affinity isoforms of the IL-2 receptor (although a tiny minority of the receptors will be high affinity receptors) and the therapy therefore involves high dose i.v. administration of the protein to destroy neoplastic cells bearing any IL-2 receptor. Such therapy is liable to show high toxicity. Moreover, as a chimeric protein, the compound is liable to be immunogenetic.

- 20 20 The IL-2-PE40 was found to have only limited activity against human T-cells and alternative molecules have therefore been constructed in a search for a more active agent (Ref 6).

- 25 WO 92/20364 describes hybrid molecules containing a first portion which is a molecule capable of decreasing cell viability (especially a cytotoxin) and a second portion which is a molecule capable of specifically binding to a cytokine receptor (especially all or a binding portion of a cytokine). The second portion targets the first portion to the cytokine receptor and is exemplified as IL-2. The IL-2 portion preferably lacks IL-2 activity because the molecules will then prevent proliferation of the target cells.

- 30 30 The present invention is based in one aspect on an insight that a medicament which contains an active promoter of proliferation, for example an active IL-2, can beneficially be used to deliver pharmacologically desirable species to cells whose proliferation is not desired. For example some medicaments of the invention control or inhibit proliferation using a molecule which contains an active promoter of proliferation. Preferred embodiments are based on an appreciation that, by using the high affinity of receptor super families, it is possible to drive drugs or genetic material, for example, into specific cell lineages which are predominantly responsible for many clinical events.

In one aspect, therefore, the present invention provides a composition of matter (product, e.g. compound) comprising a proliferatively active moiety linked to a biologically active agent which agent preferentially or selectively affects proliferating cells. In a preferred aspect there is provided a biologically active agent linked to a cytokine or growth factor which has a high affinity receptor or is a molecule functionally equivalent to such a cytokine or growth factor. The link between the two domains of the product (biologically active agent and proliferatively active agent/cytokine/growth factor) is normally capable of being broken under intracellular conditions, for example by acid hydrolysis.

Unlike other vectors evaluated, or under evaluation currently, the proliferatively active moiety (or cytokine or growth factor in the preferred aspect) retains its functional activity, which can come into play once the product targets its receptor. The proliferatively active moiety binds to the receptor and is then internalised by the cell, so that each active domain of the product (the proliferatively active moiety and the biologically active agent) can perform its respective function. It is contemplated that the two domains of the product will separate intracellularly in commercially viable products, but this is not essential and the invention is not restricted to products which are intracellularly cleavable. In preferred embodiments, the events which follow binding of the proliferatively active moiety to the receptor typically include internalisation of the product (typically a fusion compound) into the cytosol (by the endosome pathway), endosome acidification (by the proton H⁺ pump mechanism), and a separation of functional domains into the receptor domain, the proliferatively active domain, and the active agent domain. Since the receptor domain and the proliferatively active domain (normally a cytokine or growth factor domain) retain their functional integrity, the proliferatively active domain (cytokine or growth factor) will trigger cell activation/division through DNA interaction (G2-M phase enrichment).

The invention provides products in which an active moiety is linked, usually but not necessarily by a covalent bond cleavable intracellularly, with a proliferatively active moiety, i.e. a moiety which causes cellular proliferation. In preferred embodiments the proliferatively active moiety has a high affinity receptor and the products can be administered at very low dosages such that the only cells which are targeted are those presenting a high affinity receptor to the proliferatively active moiety (normally a cytokine or growth factor). As already described, the product is internalised into the target cells, where the biologically active moiety is normally separated from the proliferatively active moiety.

The invention also includes in another aspect a method of treating by prophylaxis or therapy a disease or disorder involving cells bearing a high affinity receptor for, in particular, a cytokine or growth factor, comprising administering to a patient an effective amount of a product comprising an agent which is biologically active when in said cells and is linked to said cytokine or growth factor; such products and preparations containing them form a further aspect of the invention.

Additionally included in the invention is a product of the invention for use as a pharmaceutical, especially in internalising the biologically active agent into a cell having a high affinity receptor for the proliferatively active agent, cytokine or growth factor of the product.

5 Another aspect of the invention resides in the use of a product of the invention for the manufacture of a medicament for internalising the biologically active agent into a cell having a high affinity receptor for the proliferatively active agent, cytokine or growth factor of the product and optionally for stimulating lymphocyte proliferation.

10 The biologically active agent is in one preferred class of products a substance which is effective to kill or inactivate proliferating cells, such that the activities of the physiologically active agent and the proliferatively active agent are complementary in their activity. Such a biologically active agent may be an antiproliferative drug, for example an antiblastic agent or a chemotherapeutic agent which, for example, interferes with DNA replication. The proliferatively active agent in one class of products promotes lymphocyte replication. For example a construct of IL-2 and antisense DNA/RNA designed to block a retrovirus gene obtains the following effects:

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- a. in infected cells, the replicative stimulus given by the IL-2 stimulates also replication of the viral genome, resulting in stronger inhibitory activity by the antisense DNA/RNA;
- 20 b. immunostimulation of uninfected lymphocytes, with the potential benefit of increased immune surveillance.

Amongst the features of preferred embodiments are:

- 25
- the product is a combination of two existing moieties (or of moieties functionally equivalent thereto), each of which retains its function and, optionally, its entire structure (except at any covalent linkage site to the other moiety)
 - 30 • the product can be produced by chemical combination of the two moieties, for example using standard techniques
 - the product can be administered at exceedingly low dosages, so that little or no systemic toxicity results.
 - 35 • the growth factor/cytokine stimulates the immune system and the active moiety induces a therapeutic effect

- biodistribution is predictable and good
 - targeting is good
- 5 • immunogenicity is low.

It is a feature of the first aspect of the invention that the biologically active agent (normally a medicament) is linked to a proliferatively active moiety. Unlike prior art chimeric proteins containing solely the receptor-binding domain of IL-2, therefore, these products induce cellular proliferation, enabling anti-proliferative drugs to be highly effective, even at ultra-low doses in the case of proliferatively active moieties with high affinity receptors. The invention therefore enables low systemic toxicity to be achieved. An additional benefit at least in the case of IL-2 is that IL-2 induces expression of the high affinity IL-2 receptor when the relevant antigen is present.

10 15 The invention will now be illustrated by way of example with reference to certain specific cytokines, growth factors, biologically active agents and diseases. Of course, the invention is not limited to these specific features.

15 20 The cytokine may be an interleukin, for example a TNF, for example an M-CSF; an IFN, for example an FGF; an IFG; a TGF, for example a GM-CSF; an SCF; a G-GSF; or an EPO.

The cytokine is preferably a human cytokine.

20 25 The growth factor may be a haematopoietic or lymphopoietic growth factor. They are a family of glycoprotein hormones which regulate survival, proliferation, and differentiation of progenitor cells, in addition to impacting on some functional activities of mature lymphohaematological cells.

Suitable growth factors include:

- 30 Erythropoietin (Epo);
GM-CSF;
G-CSF;
SCF (Stem cell factor);
Multi-CSF (also known as Interleukin-3);
M-CSF;
35 E-CSF (or Interleukin-5);
IGF-1 (Insulin-like growth factor);
PDGF (Platelet-derived growth factor);
TGF beta2 (Transforming growth factor -beta2)

Cytokines or growth factors (or proliferative agents) may be native or a mutein representing the native molecule modified by one or more amino acid alterations (deletions, additions or substitutions). Such muteins, usable in the present invention, possess the biological activity of the native protein, in the sense of
5 having both functional affinity for the receptor (and in one class of embodiments functional affinity for the high affinity receptor) and a capability of forming, with the receptor, a product internalised by the cell presenting the receptor.

Cytokines and growth factors are preferably recombinant molecules, but may be produced by cultivating
10 cytokines or growth factor producing cell lines, for example peripheral blood lymphocytes.

In one class of embodiments, the products comprise a biologically active compound linked to a molecule which is functional to have a high affinity with a cytokine or growth factor high affinity receptor, and to form a complex with such a receptor which is internalised by the cell presenting the receptor. In a
15 particular class of products, the molecule may be a native or mutein cytokine, or a fragment thereof. In another class of products, the molecule may be a native or mutein growth factor, or a fragment thereof.

Particularly preferred is the cytokine interleukin-2 (IL-2). IL-2 is a lymphokine which is produced by normal peripheral blood lymphocytes, and induces proliferation of antigen or mitogen stimulated T-cells
20 after exposure to plant lectins, antigens, or other stimuli. IL-2 was first described by Morgan, D A., et al., Science (1976), 193: 1007-1008. Then called T-cell growth factor because of its ability to induce proliferation of stimulated T lymphocytes, it is now recognised that, in addition to its growth factor properties, it modulates a variety of functions of immune system cells in vitro and in vivo, and has been renamed interleukin-2 (IL-2).

25 Interleukin-2 may be made by cultivating human peripheral blood lymphocytes (PBL), as described, for example, in US Patent No. 4,401,756. As a preferred alternative, the IL-2 may be recombinant. Taniguchi, T. et al., Nature (1983), 302:305-310 and Devos, R., Nucleic Acids Research (1983), 11:4307-4323 have reported cloning the human IL-2 gene and expressing it in micro-organisms.

30 US Patent No. 4,518,584 describes and claims muteins of IL-2 in which the cysteine normally occurring at position 125 of the wild-type or native molecule has been replaced with a neutral amino acid, such as serine or alanine. An oxidation-resistant mutein of IL-2 which is biologically active may be prepared wherein each methionine residue of the protein from which the mutein is derived is replaced with a conservative
35 amino acid such as alanine; the methionine residue(s) is/are susceptible to chloramine T or peroxide oxidation. These IL-2 muteins possess the biological activity of native IL-2. US Patents Nos. 4,530,787 and 4,569,790 disclose and claim methods for purifying recombinant native IL-2 and muteins thereof, as well as purified forms of IL-2. The aforesaid US patents are included herein by reference.

The IL-2 mutein desala₁-IL-2 ser¹²⁵ is available commercially from Chiron B.V. of Amsterdam, Netherlands under the trade mark Proleukin®.

- 5 In the widest aspect of the invention, the biologically active agent may in principle be any compound effective to act on the functioning of a cell in which the active agent is internalised. However it is normally a species which acts selectively or preferentially on the proliferating cell, and in such embodiments is not for example a polypeptide cytotoxin such as *pseudomonas* exotoxin or diphtheria toxin. In preferred embodiments the active agent is an immunosuppressant, for example a known immunosuppressive drug. In
10 some embodiments the active compound comprises an antisense nucleotide sequence, e.g. an anti-oncogene sequence; in other embodiments the active agent is an enzyme inhibitor, e.g. an inhibitor of a viral reverse transcriptase. Alternatively the active agent may be an antitumour or other antitumour agent. In less preferred embodiments the active agent comprises a radioactive isotope.
- 15 Particularly preferred are products comprising a biologically active agent linked to a cytokine or growth factor or to a molecule functionally equivalent thereto, the biologically active agent being selected from the group consisting of antiproliferative drugs, compounds which interfere with nucleotide synthesis, radioisotopes, gene sequences and antisense nucleotide sequences, and the cytokine or growth factor having target cells capable of presenting a high affinity receptor therefor. The biologically active agent agent is
20 suitably an immunosuppressant, an enzyme inhibitor or an anti-cancer drug.

- These particularly preferred products include those in which the biologically active agent is cyclosporin, FKK 506, thalidomide, a dihydrofolate reductase inhibitor, an antiblastic drug, a platinum coordination complex, a vinca alkaloid, a purine analogue, a pyrimidine analogue, a corticosteroid, a viral reverse transcriptase inhibitor or an antisense nucleotide sequence. A specific class of products are those in which the biologically active agent is cyclosporin, a vinca alkaloid, FKK 506, thalidomide, methotrexate, azathioprine, cyclophosphamide, actinomycin D, daunomycin, doxomycin, bleomycin, a rhenium radioisotope, an yttrium radioisotope, 3'-azido-3'deoxythymidine, an antisense nucleotide sequence that binds to a viral nucleotide sequence or an anti-oncogene nucleotide sequence.
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30 In some embodiments the active agent is not a pro-drug; of course, pro-drugs are included in the invention if the metabolism required for their conversion to the active drug is located in the cytoplasm of the target cell.
35 The products of the invention preferably act only on cells presenting a high affinity receptor for the cytokine or growth factor, which are typically lymphocytes or other cells involved in the immune response. The action of a product of the invention on its target cells depends on the function of the active agent.

The biologically active agent is preferably not a polypeptide. Preferably it is a monomeric organic compound. The biologically active agent may comprise a metal.

If the biologically active agent is an organic compound, the active agent is normally covalently linked to
5 the proliferatively active moiety (especially cytokine or growth factor). Thus, the products of the invention
are often molecules. Metal-containing products normally comprise an organometallic complex consisting
of an organic member which is covalently bonded to the proliferatively active moiety.

The molecular ratio of the active agent:proliferatively active moiety in the products of the invention is not
10 critical. Thus the invention includes ratios of 1:1 or less but in some embodiments the ratio is greater than
1:1, e.g. 1:1000 or more, i.e. a plurality of active agent molecules/atoms may be bound to each
proliferatively active moiety

15 The inventive products will now be described in more detail, by way of example, with reference to
illustrative products and product classes.

Interleukin-2/Cyclosporine fusion product

20 Cyclosporine, a second generation immunosuppressive, has a highly selective capacity to inhibit activation
of T-cells (Ref 17).

Unlike the first generation immunosuppressants, such as azathioprine, methotrexate, and
cyclophosphamide, therapeutic concentrations of Cyclosporine do not cause myelotoxicity. Cyclosporine
inhibits the early cellular response to antigenic and regulatory stimuli, primarily by targeting T-
25 lymphocytes.

30 Cyclosporine induces a rapid and profound inhibition of IL-2 production by T-cells, and causes a general
reduction in the production and release of other lymphokines in response to the antigenic stimulus. At high
intracellular concentrations, Cyclosporine inhibits expression of receptors for IL-2.

35 Cyclosporine, and particularly Cyclosporine-A (Cs-A), is therefore widely used to sustain renal, hepatic,
and cardiac transplants. The use of Cs-A in autoimmune diseases is less common, due to the side effects
(both acute and delayed), which are linked to the therapeutic range of the systemic dose used, and the
duration of the therapy.

35 The major clinical toxicity of Cyclosporine is renal, and usually occurs in 25%-75% of patients treated.
The toxicity is dose-related, and is irreversible in some patients. The reduction of the Cyclosporinemia,
within the therapeutic range, results in a worsening of the underlying disease (graft rejection). Other

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systemic side-effects include hypertension, neurological toxicity, and an increased incidence of infections due to systemic immunosuppression. This general reduction in immunocompetence also increases the incidence of malignancies in transplanted patients.

- 5 In the current invention, a preferred pharmacological product comprising chemically linked IL-2 and Cyclosporine (Cs-A or analogues in the same class of drugs) provides ultra specific immunosuppression; that is, a new third generation immunosuppressive approach. The system maintains the immunosuppressive pharmacological characteristics of Cyclosporine (Cs-A or analogues) on lymphocytes, but the action is selectively directed only to those lymphocytes which can internalise the complex (IL-2/Cs) (i.e. 10 lymphocytes bearing high-affinity receptors for IL-2), which are naturally activated in the inflammatory process (transplant rejection, autoimmune disease).

Very low doses of IL-2/Cs may be administered intravenously (i.v.) or subcutaneously (sc) in order to target active compounds in vivo. This means that extremely low doses of Cyclosporine can be used, 15 because virtually all the drug is captured by the activated lymphocytes. There is therefore a lack of general toxicity (very low plasma concentrations of IL-2/Cs) despite maintaining the pharmacological properties of the active compound (very high intracellular concentrations of the immunosuppressant drug in lymphocytes). From an immunological point of view, it is therefore possible to achieve true oligoclonal immunosuppression for the first time.

20 Cyclosporine may be replaced by structurally related or unrelated compounds with similar mechanism of action, for example thalidomide or FK506.

Interleukin-2/Methotrexate product

- 25 Methotrexate, and other dihydrofolate reductase inhibitors, as a class of antineoplastic drugs, have been used to treat selected forms of autoimmune and inflammatory diseases and, in combination with Cyclosporine, for prophylaxis of graft-versus-host disease in bone marrow transplantation (Ref 17).
- 30 The immunosuppressive activity of methotrexate is achieved by inhibiting replication and the functional activity of T-cells (and possibly B-cells), as a result of a relatively selective action on DNA synthesis. The drug is also used for the treatment of severe, active rheumatoid arthritis in adults, and in severe psoriasis.

35 The clinical toxicity of methotrexate is high, and reflects the standard toxicity of chemotherapy: severe myelotoxicity, mucositis, hepatic fibrosis, cirrhosis, and pulmonary toxicity.

Administered on a long-term basis, methotrexate, like other antineoplastic drugs, increases the incidence of infections, malignancies, and sterility in the patient. IL-2/methotrexate complex acts in the same way as IL-

13

2/Cyclosporine complex. The IL-2 drives the methotrexate into activated lymphocytes, which are responsible for a given autoimmune attack, or immune rejection. These are the only cells in the body bearing the high-affinity IL-2 receptor on the membrane. Methotrexate will inactivate only these T-lymphocytes, by blocking DNA synthesis in the cells (oligoclonal immunosuppression) through intracellular dihydrofolate reductase inhibition. The remaining immune cells, and those cells not experiencing myelotoxicity, will all remain competent. As such, the patient will be able to respond to infectious stimuli. Low dose IL-2 means low dose methotrexate. Thus the pharmacological effect of methotrexate is maintained within the lymphocytes targeted by the system, but the plasma concentration of the whole complex, or of free methotrexate, is practically undetectable.

10

Interleukin-2/azathioprine product

Intracellular nucleophiles, such as glutathione, cleave the prodrug azathioprine to mercaptopurine. This purine analogue is subsequently converted into mercaptopurine-containing nucleotides that exert effects on the synthesis and utilisation of precursors of RNA and DNA (Ref 17).

15

Azathioprine has been one of the most important first generation immunosuppressants (combined with prednisone) used to sustain transplants. It has also been used for the treatment of some autoimmune diseases, such as severe and refractory rheumatoid arthritis.

20

Unfortunately, the body distribution of this potent antineoplastic compound (i.e. its lack of specificity towards immune cells) results in high toxicity, including myelotoxicity (leukopenia and thrombocytopenia), nausea, vomiting, hepatic veno-occlusive disease, and toxicity at the germinal lineage (hypo-amenorrhea and hypo-azoospermia).

25

After the administration of the molecular complex IL-2/azathioprine at low doses (parenterally), the prodrug azathioprine is driven only into activated lymphocytes which bear the high affinity receptor for the cytokine IL-2. These cells (mainly T-cell lineage) are the major cells responsible for the immune attack during a given immune pathological event (transplant rejection or autoimmune disease). Only in these cells will the active compound mercaptopurine reach a pharmacological concentration, and be able to block the cell in exerting oligoclonal immunosuppression.

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The high specificity of this therapeutic approach significantly reduces the total dose of the antineoplastic compound administered in vivo, virtually minimising or abrogating drug induced toxicity.

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Interleukin-2/antitumur antibiotics (actin mycin D, daunorubicin, dox rubicin, bleomycin and all related comp unds).

This therapeutic complex includes the most potent antitumour agents (antiblastic drugs) widely used in the treatment of a number of malignancies.

The intracellular mechanisms of action include an interaction with DNA and RNA at several molecular
5 levels, resulting in cell death when the intracellular concentration overcomes natural mechanisms of DNA-repair and cell recovery. (Ref 17).

The pattern of clinical toxicity is extremely varied, and can be severe. All normally replicating cells in the
body will experience some damage due to a lack of specificity. These compounds circulate throughout the
10 body after administration, and interact with most cells.

Using the complex IL-2/antitumour antibiotic, administered at very low doses parenterally, the total amount
of active compound (the antitumour antibiotic) will be captured exclusively by activated lymphocytes,
present in the body as a result of autoimmune disease, or during graft rejection. The intracellular
15 concentration of the active compound in the target cells (predominantly activated T-cells) will reach a
pharmacological level which will enable it to block or kill those cells only. The very low plasma
concentration of the complex, free IL-2 or free antitumour antibiotic, is heavily diluted in litres of volume
distribution, with insignificant effects on the non-target systems.

20 Platinum coordination compounds, vinca alkaloids, purine analogues, pyrimidine analogues and
corticosteroids can form complexes with IL-2 in a similar fashion to the previously mentioned active
agents. Exemplary vinca alkaloids include Vindesine, Vinorelbine and Vinleurosine, amongst others.

Interleukin 2/radioactive isotopes product (Rhenium, Yttrium)

25 In these complexes, the active agent linked to IL-2 is a radioactive, cytotoxic isotope. IL-2 radiolabelled
with iodine has been used for diagnostic purposes, but it is novel to link cytotoxic isotopes (those isotopes
which kill cells using a microscopic high-energy radiation field) to IL-2 in order to drive the radiation field
into target cells (T-cells). Such specific radioimmunological treatment *in vivo* may be used to target an
30 immune attack in the inflammatory microenvironment during the autoimmune pathological process, or
during rejection of a transplanted organ.

Interleukin-2/AZT product

35 In this case, the main target is the Human Immunodeficiency Virus (HIV) infection.

AZT (Zidovudine, 3'-azido-3'-deoxythymidine), like other similar antiretroviral compounds in experimental
evaluation for HIV infection, is an active drug against HIV-1 retrovirus.

AZT is phosphorylated intracellularly by enzymes to the corresponding deoxynucleoside triphosphate derivative which inhibits viral reverse transcriptase. Its antiviral selectivity is due to its greater affinity for reverse transcriptase than for human DNA polymerases (Ref 16).

5

Its use is widely applied to HIV infected patients in order to reduce the replication of the HIV virus in CD4 lymphocytes (a particular subpopulation of lymphocytes which are the main target of the HIV retrovirus), and to delay the onset of systemic immunodeficiency which leads to AIDS disease.

- 10 The concentration of this drug in the body must be maintained at a high level in order to block as much virus replication as possible during a long period of treatment. This leads to mild to severe side-effects due to penetration of AZT into cells other than those in which the HIV virus is naturally present after the infection. The side effects are mainly myelotoxicity and immunosuppression.
- 15 Using Interleukin-2/AZT (or other anti-HIV compounds), the active drug will be captured and internalised only in those lymphocytes bearing high affinity receptors for IL-2, thus reducing the volume of distribution, and the interaction of the active antiretroviral compound with other cells.

In this specific case - HIV infection - the rationale for using this complex is enhanced by a unique state of the disease. The intracellular division of the complex of IL-2 (the active compound to promote lymphocyte replication) and the antiretroviral drug (the active compound to block virus replication) provides the basis for a synergistic phenomenon: (a) immunostimulation of lymphocytes which are not HIV infected, with a potential benefit in terms of increasing the general host immune surveillance in these immunocompromised patients (the active antiretroviral compound, which accumulates in the cells, is dangerous for the HIV genome but is rather inert for the cell biochemistry), and (b) in HIV infected lymphocytes, the replication stimulus, provided by IL-2, promotes the replication stimulus for the HIV genome, resulting in increased antiretroviral activity by the antiretroviral drug which is presented into the HIV-infected lymphocytes concurrently.

30 **Interleukin-2/antisense product**

IL-2/antisense fusion products are useful for introducing specific antisense sequences (oligonucleotides) into lymphocytes bearing the IL-2 receptor.

- 35 Antisense oligonucleotides are short synthetic single strand nucleotides containing sequences complementary to target mRNA or DNA. It is possible to create an antisense compound which blocks specific DNA or RNA sequences related to the HIV genome. What was hitherto unknown, however, was the means to introduce these particular sequences *in vivo*. Using cytokines (e.g. IL-2) which internalise

into target cells, an antisense compound for a given tract of the HIV genome (e.g. genome coding for an envelope protein, or for the enzyme transcriptase) may be introduced specifically into the lymphocytes of a given patient, using a pharmacological strategy, such as IV administration, for example.

- 5 IL-2/antisense products may also be used to introduce antisense compounds or antioncogenes into the T-cell lineage affected by neoplastic transformation, where gene mutation, or oncogene hyperexpression is known.

Treatable Diseases

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The IL-2/active compound fusion products are useful for diseases for which the lymphocyte is mainly involved in tissue damage, and the resultant development of a given disease entity.

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In essence, Interleukin-2 high-affinity receptor-directed immunosuppressive therapy acts pharmacologically, but only on recently activated lymphocytes (particularly T-cells), which bear this structure on the cellular membrane. The activation signal is absent from the surface of resting T-cells and all other non-lymphoid tissues. As such, very low doses of cytokine/immunosuppressive drugs can be targeted.

20

Since the receptor is only transiently expressed during the brief proliferative phase, when lymphocytes respond to antigen stimuli (autologous-antigen in the case of autoimmune diseases, and heterologous-antigens in the case of transplantation), it is possible to achieve selective *in vivo* immunosuppression, directed solely towards activated lymphocytes (oligoclonal immunosuppression). This pharmacological action is totally different from the general immunosuppression action exerted by conventional immunosuppressive drugs.

25

Diseases which can benefit from this approach include autoimmune diseases, transplant rejection, HIV-infection, and lymphoproliferative diseases.

30

Autoimmune Diseases

Autoimmune diseases are a wide variety of disorders with a common pathogenic pathway: immune attack on target organs due to abnormal recognition of tissue antigens, and/or cellular antigens, by the immune system, particularly by T-lymphocytes (17).

35

This immune attack is implemented by a network of T-cell-mediated cytotoxicity, humoral autoimmune antibodies produced by B-lymphocytes, complement activation and consumption, and finally by tissue

damage. The central role of the abnormal activation of the T-lymphocytes lineage in all autoimmune diseases is well recognised.

The clinical disorders under this heading and their target organs include the following:

5

1. Autoimmune diabetes mellitus (Type I diabetes) ---> endocrine pancreas
2. Autoimmune thyroiditis (Hashimoto and others) ---> thyroid
3. Autoimmune hepatitis (chronic active hepatitis) ---> liver
4. Rheumatoid arthritis ---> synovial/joints/viscera
- 10 5. Autoimmune Nephritis (glomerulonephritis) ---> kidney
6. Uveitis (Behcet's syndrome) ---> eye
7. Multiple sclerosis ---> CNS/PNS
8. Sjogren syndrome ---> saliva glands
9. Scleroderma ---> skin/viscera
- 15 10. Dermatopolymyositis ---> skin/muscle/viscera
11. Systemic Lupus Erythematosus(SLE)---> viscera/skin/hematopoieses/mucose
12. Autoimmune hemolytic anaemia ---> erythrocyte
13. Idiopathic thrombocytopenic purpura (ITP) ---> platelet
14. Autoimmune neutropenia ---> neutrophil
- 20 15. Vasculitis ---> vessels
16. Crohn's disease ---> bowel
17. Ulcerative colitis ---> bowel
18. Coeliac disease ---> bowel
19. Psoriasis ---> skin/joints/viscera
- 25 20. Sarcoidosis ---> lung/viscera/skin
21. Atopic syndromes

In the majority of these pathological manifestations, there is a pathogenic lymphocyte-mediated reaction, and cytotoxicity.

30

The evidence to support the role of T-cells in the pathogenesis of specific disease and progression of targeted tissue damage is substantial. In most of the diseases listed above, CD4 cells (a cytotoxic subset of T-cells) are the dominant T-cell phenotype in the target tissues. T-cells express several activation markers. Experimentally, there is evidence that autoimmune diseases improve when T-cell targeted intervention occurs, as in thoracic duct drainage, total lymphoid irradiation, and administration of Cyclosporin. Furthermore active autoimmune disease is generally less severe in AIDS patients who have CD4 cytopenia.

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Most autoimmune diseases are treated by attempting to reduce the function of the immune system using immunosuppressive and anti-inflammatory drugs. This therapeutic strategy is conducted in a non-specific way, resulting at times in iatrogenic toxicity and a failure to control the overall disease process.

5 Lymphocytes, responsible for the acute phase of a given autoimmune attack, all bear the high-affinity IL-2 receptor on the membrane. They are antigen-activated, or cytokine-activated, lymphocytes with a high avidity for IL-2.

The parenteral administration of very-low doses of IL-2/ immunosuppression products enables the use of
10 IL-2 as the vector of pharmacologically active drugs to exert immunosuppression. The IL-2/immunosuppressant products selectively bind to, and interact with, only those cells bearing the high-affinity receptor of IL-2. This means that immune cells, responsible for tissue damage and disease progression, are inactivated selectively, potentially curing patients with chronic diseases, or reducing their relapse rate.

15 Using this novel invention, it is possible to achieve highly-specific immunosuppression (so-called oligoclonal deletion), maximizing the efficacy of immunosuppressive drugs, and abrogating most toxicities.

IL-2, used alone at low or very low doses, has already been shown to be totally safe, and can be
20 administered for long periods. Immunosuppressive drugs, used alone at ultra low concentrations, have no efficacy (or toxicity). However, linked together, the two compounds achieve high concentrations only where needed (intracellularly).

Recombinant proteins, for example recombinant IL-2 and other recombinant cytokines and growth factors,
25 usually have low immunogenicity and good tissue distribution. After parenteral administration, every tissue compartment in the body is exposed, including all lymphocytes (circulating lymphocytes, lymphocytes in the tissues, and lymphocytes in the lymph nodes).

Very-low doses of immunosuppressive or antiblastic drugs, such as Cyclosporine, cytostatic agents, and
30 others mentioned above, all result in a reduction of "standard" toxicities, normally experienced by the patient (myelotoxicity, renal dysfunction, metabolic disorders, brain dysfunction, cardiovascular problems, infections, opportunistic infections and malignancies).

Transplantation

35

The acute or chronic rejection of a transplanted organ is related to heterologous antigens (antigen specific to the donor transplanted organ(s)) presenting to host T-cells. Following antigen presentation and recognition, immune cells enter into a proliferative phase, during which the high-affinity receptor for IL-2

is expressed. This leads to activation of the cytotoxic process, and to damage and subsequent failure of the transplanted organ.

The use of IL-2 as a vector to target immunosuppressive drugs achieves longevity of transplanted organs

5 without the associated toxicity of conventional immunosuppressive therapy (acute, delayed, and long term).

Allogeneic bone marrow transplantation (ABMT) is used to treat and cure leukemias (both lymphoid and myeloid), thalassemia, and solid tumours. The products of the invention have the potential to reduce the incidence of Graft-Versus-Host-Disease (GVHD), which is the reaction of the donor immune system 10 against tissue antigens of the host, without compromising the global immune-system (of graft origin). As a result, there could be a reduction in mortality rate due to GVHD (currently in excess of 40%), a reduction in infectious complications, and a positive anti-tumour effect on minimal residual disease (the so-called Graft-Versus-Leukaemia effect).

15 HIV-Infection

The administration of low-dose IL-2/AZT fusion product (or IL-2/antiretroviral analogue, or IL-2/antisense specific to HIV-genome fraction) exerts *in vivo* an immunostimulatory effect on HIV-negative lymphocytes bearing the high-affinity receptor. The anti-retroviral compound is introduced into the 20 cytoplasm of HIV-infected lymphocytes (CD4 cells), leading to a selective destruction of infected cells, without impacting on the normal reactive lymphocytes which are stimulated.

Lymphoproliferative diseases (Lymphoblastic leukaemia and lymphomas)

25 Using an IL-2/cytotoxic fusion complex, it is possible to selectively kill the neoplastic lymphoid lineage expressing the high-affinity IL-2 receptor, without inducing any critical systemic toxicity on non-lymphoid compartments.

Products containing growth factors or cytokines other than IL-2 may be used in the therapies described 30 below:

TNF- α has as its target cells macrophages and osteoclasts. TNF- α /blocking compound products may be used to insert into macrophages a blocking compound (which blocks cellular function and/or kills the cell). Such products potentially provide an important tool in some pathological conditions, e.g. advanced solid 35 cancers where macrophage hyperstimulation and activation is responsible for cachexia and tumour progression, monocyto-macrophage neoplasms (e.g. histiocytosis), transplant rejection and GVHD, autoimmunity, and neurological degenerative diseases (the TNF receptor in its extracellular domain is similar to nerve Growth Factor receptor).

M-CSF (macrophage colony stimulating factor) may be used to form products containing blocking compounds, useful for treating the same classes of conditions as TNF- α /blocking compound products.

5 M-CSF is also responsible for microglial proliferation in the CNS. Other potential applications are therefore in some degenerative diseases of the CNS, such as Alzheimer's syndrome, and in bone diseases.

The products IFN/active compound (IFN- α , - β or - γ) may be used to modify the function of activated lymphocytes, macrophages, endothelial cells and fibroblasts, or to incapacitate them in different 10 pathological conditions, such as in HIV-infection (AIDS), and fibroblast-related diseases such as scleroderma.

Potential applications of FGF products include their use as antiangiogenic factors in solid cancers, and to block hyperactivation of fibroblasts in scleroderma. The invention enables the preparation of FGF products 15 capable of acting as an antagonist in relation to the cell types listed earlier in this specification as having FGF high affinity receptor when activated.

IFG/antiblastic products may be used to treat breast cancer. Due to the presence of the high affinity receptor in CNS (neuroglia), it could also be used in some degenerative neurological disorders.

20 TGF β products have applications similar to those of FGF and IFG fusion products.

GM-CSF/active compound fusion products may be used to selectively kill myeloid blasts responsible for myeloid leukemias. G-CSF products have similar pharmacological activity to the GM-CSF products, but 25 bind to a different high-affinity receptor present only in multipotent stem cells in the bone marrow.

Epo fusion products may be used for diseases such as polycythemia and erythroleukemia, for example.

30 Epo/gene sequence fusion products, in which the DNA fraction is the normal gene for haemoglobin beta-chain, may be used for introducing the normal gene into the erythroid lineage in patients affected by beta-Thalassemia. In this genetic disease, the abnormal gene, coding for a non-functional haemoglobin beta-chain, is present in the erythroblastic progenitors in the bone marrow. The insertion of the normal gene, through the Erythropoietin vector, selectively into the bone marrow erythroblastic lineage, represents true 35 in vivo gene-therapy, to potentially cure patients with this disease. The same consideration applies to another genetic haemoglobin disease: sickle cell anaemia.

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IL-6/fusion products may be used to block cells, having IL-6 high affinity receptors, which are involved in multiple myeloma, osteoclastic hyperactivation (metastasis to the bone), cancer-related bone lesions and osteoporosis.

- 5 Turning specifically to the class of products containing a growth factor (or its functional equivalent in terms of receptor affinity), the following fusion products and disease targets can be considered (where "CTX" denotes a cytotoxin):

Fusion product and related disease target for cytotoxics

- 10 1. Epo/CTX in erythroleukemia (Di Guglielmo Syndrome, also known as M6 Leukemia in FAB classification)
2. GM-CSF/CTX in Chronic Myeloid Leukemia (CML), Acute Myeloid Leukemia (AML)
3. G-CSF/CTX in CML, AML
4. SCF/CTX in AML, Acute Lymphoblastic Leukemia (ALL) and CML accelerated phase (blastic crisis)
- 15 5. IL-3/CTX in ALL, CLL, CML and AML
6. M-CSF/CTX in Acute Monocytic Leukemia (FAB M5a and M5b) and Monomyelocytic Leukemia (FAB M4)
7. M-CSF/CTX in Wegener's Disease, Granulomatosis, Inflammatory Breast Cancer, Giantcellular vasculitis, Sarcoidosis, histiocitic necrotizing lymphadenitis (Kikuchi's disease)
- 20 8. IL-5/CTX in eosinophilic syndromes (Wegener, Polymyositis, Granulomatosis, systemic allergic skin reactions, parasitosis)
9. GM-CSF (or) G-CSF (or) SCF (or) IL-3/CTX in Myelodysplastic Syndromes (MDS)
10. IGF-1/CTX in breast cancer
- 25 11. TGF/CTX in malignant transformations of the bone (osteosarcoma, chondrosarcoma, fibrosarcoma) and fibrodysplastic syndromes (scleroderma)
12. TGF/CTX in anti-angiogenesis (adenocarcinomas).

Growth factor fusion products may be used as active vectors in targeting genes. In the following discussion, the same growth factors are as above considered, plus Interleukin-2. A critical point is the fact that these vectors are not only active as transporters of genes, but also promote the rearrangement of DNA, in addition to opening DNA chains in target cells. They are therefore ideal for integrating genes both in vitro and in vivo. No other system available has a comparable bi-modal activity, and without the associated risks of viruses (used currently as vectors). Suitable fusion products and disease targets include:

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Fusion product and related disease target for genes

1. Epo/Hb-beta-gene in Thalassemia

2. Epo/SS-wild gene is Sickle Cell Anemia (or Sickle Cell Disease)
3. GM-CSF/ABL_BCR gene in Chronic Myeloid Leukemia (CML) Philadelphia + (Ph+) and its
5 acellerated phase (Blastic crisis)
4. G-CSF/ABL_BCR gene in Chronic Myeloid Leukemia (CML) Philadelphia + (Ph+) and its
acellerated phase (Blastic crisis)
- 10 5. SCF/ABL_BCR gene in Chronic Myeloid Leukemia (CML) Philadelphia + (Ph+) and its
acellerated phase (Blastic crisis) and AML Ph+ (rare subform of AMLs)
6. IGF-1/P53 tumor-suppressor gene in breast adenocarcinoma
- 15 7. IGF-1/antisense HER-2Neu in breast adenocarcinoma
8. IL-2 (or) IL-3 (or) GM-CSF/functional genes in congenital immunodeficiencies (usually these
immunodeficiencies are inherited as poligenic defects: severe combined immunodeficiencies, Di
George's syndrome, Nezelof's syndrome, Ataxia-teleangiectasia, X-linked gammaglobulinemia)
- 20 9. IL-2/functional gene in selective deficiency of T-lymphocyte function such as inherited purine
nucleoside phosphorylase deficiency (PNP syndrome)
10. M-CSF/functional gene in congenital macrophage enzymatic monogenic deficiencies usually
25 present in lysosomal storage diseases. Lysosomal storage diseases include most of the lipid
storage disorders, the mucopolysaccharidoses and glycoprotein storage diseases which are
characterised by mono-enzymatic defects (beta-galactosidase, beta-glucocerebrosidase deficiency
in Gaucher's disease, alpha-fucosidase deficiency in Fucosidosis, ceramidase deficiency in
Farber's disease and hexosaminidase-A deficiency in Tay-Sachs syndrome). All these serious
30 congenital conditions may have their onset in infantile, juvenile and adult age.

M-CSF/wild gene coding for a functional enzyme selected on the basis of the specific deficiency, once integrated into macrophages and transcribed into protein, could compete with the non-functional protein and repair the defect *in vivo*.

23

The active agent and the cytokine or growth factor, or its equivalent are suitably linked using a multifunctional (e.g. bifunctional) linker which reacts with respective functional groups on the active agent and the peptide hormone (or equivalent). If the active agent is a radioactive isotope, a linker-chelator may be used, which forms a covalent bond with the cytokine or growth factor or its equivalent, and chelates the isotope. In some embodiments the two constituent parts are linked by an intracellularly cleavable link. In other embodiments, the link is intracellularly stable.

5 The preparation of products comprising a polypeptide linked to another moiety is well known, as for example in the case of fusion proteins, and the skilled person will therefore require no elucidation of 10 preparatory techniques. In general terms, suitable linkers are multifunctional, and especially bifunctional compounds capable of reacting with at least two polypeptide molecules.

15 One exemplary technique involves the use of acid-cleavable reagents for interlinking two polypeptides. Such acid-cleavable linker reagents, based on orthoester, acetal and ketal functionalities, have been described previously (Ref 18), and are bifunctional compounds whose hydrolytic rate constants increase as the pH decreases. The crosslinkers react with the proteins via heterobifunctional groups (e.g. maleimide or N-hydroxysuccinimide ester) or homobifunctional groups (e.g. bis-maleimide or bis-succinimidyl).

20 Three particular cross-linking agents which may be used are:

1. Disuccinimidyl suberate. This is a homo-bifunctional cross-linking reagent, containing the N hydroxy succinimide ("NHS") ester reactive group, which is reactive towards amino groups. The chain of the cross-linking reagent is non-cleavable.

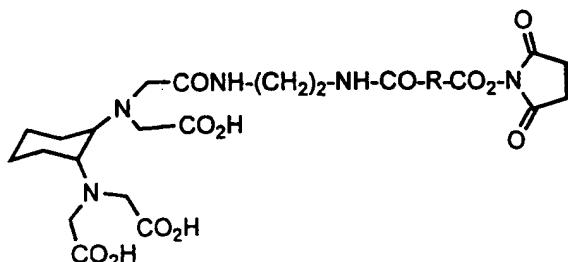
25 2. Ethylene glycabis[succinimidyl succinate]. This too is a homo-bifunctional cross-linking reagent, containing the NHS ester reactive group, which is reactive towards amino groups. The chain of the cross-linking reagent is cleavable.

30 3. Succinimidyl 6-[3-(2-pyridylthio)-propionamido] hexanoate. This is a hetero-bifunctional cross-linking reagent, containing the pyridylthio and NHS ester reactive groups, which are reactive towards sulphhydryl and amino groups. The chain of the cross-linking reagent is cleavable.

35 Products in which the proliferatively active moiety (especially cytokine or growth factor) and a biologically active agent are acid-cleavably linked benefit from the potential advantage that the product is cleaved in the endosome to release the active agent in free form. The free agent will potentially be more efficacious in its intracellular action than the linked agent.

In the case of nucleotide (DNA or RNA) active agents, for example for gene targeting, cytokines or growth factors can be linked covalently to the nucleotide. The resultant product may be mixed with the nucleotide to form a protein/DNA complex.

- 5 The literature describes a number of techniques for linking radioisotopes to proteins, typically by using bifunctional linker-chelating agents. For example, the reader is referred to the textbook "Laboratory Techniques in Biochemistry and Molecular Biology", Volume 19, edited by R. H. Burdon and P. H. van Knippenberg, published by Elsevier and to Chapter 3 ("Peptide-Carrier Conjugation") of the textbook "Synthetic Polypeptides as Antigens" by M. H. V. Van Regenmortel et al, published by Elsevier (1988);
 10 these publications contain guidance also on linking peptides to substances other than radioisotopes. Gestin et al (Ref 19) describe the preparation of such linker-chelating agents comprising trans-1,2-diaminocyclohexane-N, N, N', N' -tetraacetic acid as the liquid. Gestin describes the preparation of 5 linker-chelates of the general formula



- 15 where R = -(CH₂)₆- , -(CH₂)₂-O₂C-(CH₂)₂-CO₂-(CH₂)₂- , -CH₂-S-CH₂- , -(CH₂)₂-S-(CH₂)₂ or -(CH₂)₂-S-S-(CH₂)₂

The linker-chelates are reacted with the cytokine or growth factor, or its functional equivalent, and then used to chelate a radioisotope.

- 20 A review by T J McKearn (Ref 20) discusses techniques for linking radioisotopes to proteins, typically by means of linker-chelators. The techniques discussed by McKearn are in the context of radioimmunodetection but are applicable also to the products of this invention.
- 25 Quadri et al (Ref 21) describe the preparation of stable and labile linkages between polypeptides (antibodies) and chelators. The chelator used was DTPA (diethylene triaminepentaacetic acid). For synthetic purposes, the derivative aminobenzyl-DTPA (ABDTPA) was prepared and the amino group used to couple the DTPA with a suitable linker. As labile linkers, ethylene glycol bis (succinimidylsuccinate) (EGS) and disuccinimidyl tartarate (DST) are used. The EGS or DST are mixed, e.g. in equimolar ratio, with ABDTPA, in an anhydrous solvent such as DMSO. The polypeptide suitably in anhydrous solvent (e.g. DMSO) is added to the resultant linker-chelator to form the polypeptide-linker-chelator molecule. In another technique, described by Quadri, the EGS or DST in the preceding procedure is replaced by the

25

relatively stable hydrocarbon linker DSS (disuccinimidylsuccinate). A third technique, described by Quadri, is to react ABDTPA with thiophosgene (80% solution in CHCl₃) to make isothiocyanatobenzyl-DTPA (ITCB-DTPA). The ITCB-DTPA was reacted with amino groups of the protein in aqueous solution.

- 5 Linkers suitable for conjugating cytokines and growth factors to biologically active agents are obtainable from Pierce & Warner (UK) Limited, 44 Upper Northgate Street, Chester CH1 4EF, UK, whose literature provides further information.

Administration

10

The IL-2 and other cytokines or growth factors may be administered parenterally, in an amount of from 10.000 - 1.000.000 International Units, and suitably by intravenous, intramuscular or subcutaneous injection (less than 1 µg to 0.1 mg of recombinant protein), to give very low plasma concentrations. For example, the plasma IL-2 concentrations may be close to the dissociation constant (KD) of the 15 concentration of IL-2 that saturates 50% of the IL-2 high-affinity receptor isoform. Furthermore, this range of dose is generally without systemic adverse side effects.

The products of the invention may be formulated as human or veterinary pharmaceutical formulations in practice comprising a pharmaceutically acceptable diluent carrier or excipient.

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The formulations may be in the form of solutions or suspensions. The formulations are suitable for parental (e.g. iv or sc) administration but, oral formulations are not excluded.

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It will be seen, therefore, that the invention provides a product comprising a biologically active agent linked to a moiety which is a peptide hormone, which has a high affinity receptor, or is a molecule functionally equivalent to the peptide hormone in relation to the high affinity receptor. The peptide hormones include cytokines and growth factors. As examples may be mentioned molecules comprising a domain functionally equivalent to a growth factor (e.g. all or part of a native growth factor) and a cytotoxin domain.

30

As used herein the word "comprises" is not exclusive, i.e. it indicates that the subject of the verb need not consist only of its object but may include the object of the verb and one or more additional elements. Cognate expressions are to be construed accordingly.

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CLAIMS

1. A product comprising a proliferatively active moiety linked to a biologically active agent which agent preferentially or selectively affects proliferating cells.

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2. A product of claim 1, wherein the link between said agent and said moiety is intracellularly cleavable.

3. A product of claim 2, wherein the link is cleavable by acid hydrolysis.

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4. A product of any of claims 1 to 3, wherein target cells of the proliferatively active moiety have high affinity receptors therefor.

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5. A product of claim 4, wherein the proliferatively active moiety is a cytokine or growth factor or a molecule functionally equivalent thereto.

6. A product of claim 5, wherein the moiety is a cytokine or a molecule functionally equivalent to a cytokine.

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7. A product of claim 6, wherein the cytokine is an IL, a TNF, and M-CSF, an IFN, an FGF, an IFG, a TGF, a GM-CSF, an SCF, a G-CSF or an Epo.

8. A product of claim 7, wherein the IL is IL-2 or IL-6, the TNF is TNF- α , IFN is IFN α , IFN- β or IFN- γ and the TGF is TGF β .

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9. A product of claim 5, wherein the moiety is a growth factor or a molecule functionally equivalent to a growth factor.

10. A product of claim 9, wherein the growth factor is:

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Erythropoietin (Epo);

GM-CSF;

G-CSF;

SCF (Stem cell factor);

Multi-CSF (also known as Interleukin-3);

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M-CSF;

E-CSF (or Interleukin-5);

IGF-1 (Insulin-like growth factor);

PDGF (Platelet-derived growth factor);

TGF beta2 (Transforming growth factor-beta2).

11. A product of claim 5 wherein the cytokine or growth factor is a human cytokine or growth factor and said molecule is functionally equivalent thereto.

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12. A product of any of claims 5 to 10, wherein said moiety is a recombinant human cytokine or growth factor, optionally modified by one or more amino acid alterations.

13. A product of claim 12, wherein the recombinant human cytokine is recombinant IL-2.

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14. A product of claim 13, wherein the recombinant IL-2 is desala₁-IL-2 ser₁₂₅.

15. A product of any of claims 1 to 14, wherein the biologically active agent is an antiproliferative drug, a compound which interferes with nucleotide synthesis, a gene sequence or an antisense nucleotide sequence.

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16. A product of claim 15, wherein the biologically active agent is cyclosporin, FKK 506, thalidomide, a dihydrofolate reductase inhibitor, an antiblastic drug, a platinum coordination complex, a vinca alkaloid, a purine analogue, a pyrimidine analogue, a corticosteroid, a viral reverse transcriptase inhibitor or an antisense nucleotide sequence.

17. A product of any of claims 1 to 14, wherein the biologically active agent is an immunosuppressant, an enzyme inhibitor, an anti-cancer drug or a radioisotope.

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18. A product of any of claims 1 to 14, wherein the biologically active agent is cyclosporin, a vinca alkaloid, FKK 506, thalidomide, methotrexate, azathioprine, cyclophosphamide, actinomycin D, daunomycin, doxomycin, bleomycin, a rhenium radioisotope, an yttrium radioisotope, 3'-azido-3'deoxythymidine, an antisense nucleotide sequence that binds to a viral nucleotide sequence or an anti-oncogene nucleotide sequence.

19. A product of any of claims 1 to 18, wherein the ratio of biologically active agent to proliferatively active moiety is greater than 1:1.

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20. A product comprising a biologically active agent linked to a cytokine or growth factor or to a molecule functionally equivalent thereto, the biologically active agent being selected from the group consisting of antiproliferative drugs, compounds which interfere with nucleotide synthesis, radioisotopes, gene sequences and antisense nucleotide sequences, and the cytokine or growth factor having target cells capable of presenting a high affinity receptor therefor.

21. A product of claim 20, wherein the cytokine or growth factor is as defined in any one of claims 6-8 or 10-14.
- 5 22. A product of claim 20 or claim 21, wherein the biologically active agent is an immunosuppressant, an enzyme inhibitor or an anti-cancer drug.
- 10 23. A product of claim 20 or claim 21, wherein the biologically active agent is cyclosporin, FKK 506, thalidomide, a dihydrofolate reductase inhibitor, an antiblastic drug, a platinum coordination complex, a vinca alkaloid, a purine analogue, a pyrimidine analogue, a corticosteroid, a viral reverse transcriptase inhibitor or an antisense nucleotide sequence.
- 15 24. A product of claim 20 or claim 21, wherein the biologically active agent is cyclosporin, a vinca alkaloid, FKK 506, thalidomide, methotrexate, azathioprine, cyclophosphamide, actinomycin D, daunomycin, doxomycin, bleomycin, a rhenium radioisotope, an yttrium radioisotope, 3'-azido-3'deoxythymidine, an antisense nucleotide sequence that binds to a viral nucleotide sequence or an anti-oncogene nucleotide sequence.
- 20 25. A product of any of claims 20 to 24, wherein the ratio of biologically active agent to proliferatively active moiety is greater than 1:1.
- 25 26. A product which is a molecule comprising first domain which comprises an IL-2 sequence functional to be recognised by high affinity IL-2 receptors and to promote proliferation bonded to a second domain which comprises a biologically active agent selected from the group consisting of antiproliferative drugs, compounds which interfere with nucleotide synthesis, radioisotopes, gene sequences and antisense nucleotide sequences.
- 30 27. A product of any of claims 1 to 18, wherein the ratio of second domain to first domain is greater than 1:1.
- 28 A product of any of claims 1 to 27 for use as a pharmaceutical.
- 35 29. The use of a product of any of claims 1 to 28 for the manufacture of a medicament for treating by therapy or prophylaxis a disease or disorder involving cells bearing a high affinity receptor for a proliferatively active moiety.

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30. The use of claim 29, wherein said moiety is IL-2 or a molecule functionally equivalent thereto and the disease or disorder is an autoimmune disease, transplant rejection, graft-versus-host-disease, a retroviral disease or a lymphoproliferative disease.

5 31. A pharmaceutical formulation, comprising a product of any of claims 1 to 28 formulated for pharmaceutical use.

32. A pharmaceutical composition, comprising a product of any of claims 1 to 28 and a pharmaceutically acceptable diluent, excipient or carrier.

10 33. The use of a product of any of claims 1 to 28 for the manufacture of a medicament for internalising the biologically active agent into a cell having a high affinity receptor for the proliferatively active moiety, cytokine or growth factor of the product and optionally for stimulating lymphocyte proliferation.

15 34. A method of treating by therapy or prophylaxis a disease or disorder involving cells bearing a high affinity receptor for a proliferatively active moiety, comprising administering to a patient an effective amount of a product of any of claims 1 to 28, which product includes a proliferatively active moiety having high affinity for said receptor.

20 35. A product comprising a biologically active agent linked to a moiety which is proliferatively active.

36. A product comprising a biologically active agent linked to a moiety which is a peptide hormone, which has a high affinity receptor, or is a molecule functionally equivalent to the peptide hormone in relation to the high affinity receptor.

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